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Gamma irradiation during gametogenesis in young adult zebrafish causes persistent genotoxicity and adverse reproductive effects

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Abstract

The biological effects of gamma radiation may exert damage beyond that of the individual through its deleterious effects on reproductive function. Impaired reproductive performance can result in reduced population size over consecutive generations. In a continued effort to investigate reproductive and heritable effects of ionizing radiation, we recently demonstrated adverse effects and genomic instability in progeny of parents exposed to gamma radiation. In the present study, genotoxicity and effects on the reproduction following subchronic exposure during a gametogenesis cycle to ^{60}Co gamma radiation (27 days, 8.7 and 53 mGy/h, total doses 5.2 and 31 Gy) were investigated in the adult wild-type zebrafish (*Danio rerio*). A significant reduction in embryo production was observed one month after exposure in the 53 mGy/h exposure group compared to control and 8.7 mGy/h. One year later, embryo production was significantly lower in the 53 mGy/h group compared only to control, with observed sterility, accompanied by a regression of reproductive organs in 100% of the fish 1.5 years after exposure. Histopathological examinations revealed no significant changes in the testis in the 8.7 mGy/h group, while in 62.5 % of females exposed to this dose rate the oogenesis was found to be only at the early previtellogenic stage. The DNA damage determined in whole blood, 1.5 years after irradiation, using a high throughput Comet assay, was significantly higher in the exposed groups (1.2 and 3-fold increase in 8.7 and 53 mGy/h females respectively; 3-fold and 2-fold increase in 8.7 and 53 mGy/h males respectively) compared to controls. A significantly higher number of micronuclei (4-5 %) was found in erythrocytes of both the 8.7 and 53 mGy/h fish compared to controls. This study shows that gamma radiation at a dose of exposure ≥ 8.7 mGy/h during gametogenesis causes adverse reproductive effects and persistent genotoxicity (DNA damage and increased micronuclei) in adult zebrafish.

Key words: zebrafish; gamma irradiation; reproduction; genotoxicity; DNA.

1 Introduction

The aquatic environment is a primary recipient of ionizing radiation as the consequence of increasing amounts of gamma emitting radionuclides from various anthropogenic and non-anthropogenic activities (nuclear accidents, nuclear power plant waste discharge, cosmic radiation, naturally occurring primordial radionuclides). Gamma radiation is a potent agent for breaking bonds in the genetic material or causing cellular damage through the induction of oxidative stress, particularly in dividing cells having high active metabolism. As such, it has the potential to induce reprotoxicity and genetic defects (Adam-Guillermin et al., 2012; Hurem et al., 2017a) and impair reproductive function in aquatic fauna (Won et al., 2015). Germ cells are the precursors of the gametes (oocytes and sperm), and due to their characteristics of rapid cell division and high active metabolism are particularly vulnerable to ionizing radiation. Ionizing radiation-induced cell damage can result in a variety of deleterious effects during the lifetime of an organism, and as germ cell damage has been found to be transmissible and inherited by future generations, such damage can also result in more long-term population effects (Kong et al., 2016).

To date, the effects of ionizing radiation on the reproductive performance in fish have only been studied following exposure to either acute (Michibata et al. 1976; Hyodo-Taguchi and Egami, 1976; Kuwahara et al., 2003) or very high chronic doses (Hyodo-Taguchi and Etoh, 1983). In addition, DNA damage was analyzed in adult fish with single high dose exposures, but not chronic exposure scenarios (Lemos et al., 2017).

Although doses in the environment tend to be lower than those used in laboratory experiments, previous studies have reported exposure of aquatic biota to high doses of ionizing radiation after nuclear accidents. In the contaminated Ural lakes (near Mayak PA) following the Kyshtym accident, in 1957 doses to fish were estimated to 30-40 mGy/day (Sazykina and Kryshev, 2003).

Furthermore, fish and other aquatic organisms in the Chernobyl reactor cooling pond accumulated doses of up to 10 Gy during the first 60 days of the accident (Hinton et al., 2007).

Studies of genotoxic and reprotoxic effects in fish from ionizing radiation exposure that covers the entire gametogenesis cycle are still scarce. The zebrafish (*Danio rerio*) has proven to be a good vertebrate model to assess reproductive effects (Hoo et al., 2016; Laan et al., 2002) due to its developmental and physiological advantages such as a short reproduction cycle, high fecundity, transparent embryos and a high degree of similarity with other vertebrates. A pair of adult zebrafish can reproduce approximately two times per week over its breeding cycle, and yield 200 to 300 eggs at each spawning. In addition, the maximal reproductive capacity in zebrafish is known, and can be achieved by young sexually mature fish between three and six months of age (Skidmore, 1965). The United Nations Scientific Committee for the Effects of Atomic Radiation 1996 report stated that aquatic organism populations including fish would not be negatively affected by a chronic dose rate of 400 μGy/h (0.4 mGy.h), although a reduction of spermatogonia at this dose rate can be found (UNSCEAR, 1996). However, the span of dose rates known to inflict damage to the reproductive organs is quite broad as a total dose of 10 Gy caused minimal effects on the maturation of oocytes in fish (UNSCEAR 1996).

The present work assessed the effects of subchronic gamma radiation exposure (27 days, ^{60}Co, dose rates 8.7 and 53 mGy/h, total 5.2 and 31 Gy) in adult zebrafish during a gametogenesis cycle on the overall health, reproduction, and genotoxicity. In order to determine whether reproductive function is impaired in later life following radiation exposure, effects on reproduction were evaluated both one month and one year after irradiation. Histopathological examination of the gonads was performed in order to determine possible deleterious reproductive effects in irradiated adults, while the genotoxic effects in the form of DNA damage and the number of micronuclei (MN) in red blood cells were assessed in both male and female zebrafish one year after gamma irradiation.

2 Materials and Methods

2.1 Fish husbandry

Adult zebrafish (ZF, aged 6 months) from the AB wild type strain (30 males and 30 females per exposure group) were obtained from the Zebrafish Facility at the Norwegian University of Life Sciences (NMBU). The exposure of ZF to external gamma radiation took place at the FIGARO Co-60 irradiation facility (source activity ~420 GBq) at NMBU and is schematically depicted in Fig 1. Recirculating system water was prepared from particle and active charcoal filtered reverse osmosis kept sterile by UV irradiation water of pH 7.5 and temperature 28 ± 1 °C with regular weekly or daily water changes depending on the water quality described in Hurem et al. (2017b). The light regime of 10-14 light-dark cycle (250-320 lx) was used and fish were fed dry feed Gemma Micro 300 (Skretting, Stavanger, Norway) twice a day and live artemia (Scanbur, Copenhagen, Denmark) once a day, both during and after the experimental periods.

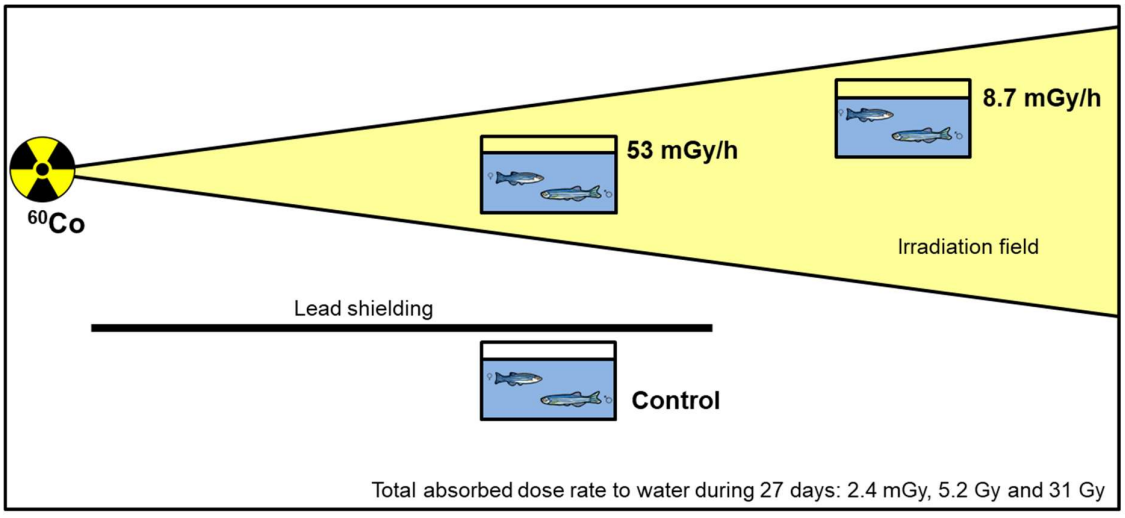


Fig 1. Schematic presentation of adult fish exposure at the FIGARO Co-60 irradiation facility at the Norwegian University of Life Sciences (NMBU). Fish were exposed in 9 L plastic aquaria, with 6 L swimming space (N = 30 males and 30 females per each aquarium). Exposure lasted for 27 days during gametogenesis, with total exposure time of 591.5 hours. A control

aquarium was placed behind lead shielding, and two aquaria at different distances to the source focus, resulting in calculated average absorbed dose rates to water of 8.7 mGy/h and 53 mGy/h, respectively, and total doses 5.2 Gy and 31 Gy, according to dosimetry described previously by Hurem et al. (2017b).

After exposure, fish were maintained according to standard operating procedures at the NMBU Zebrafish Facility until sampling for histopathology, genotoxic effects and measurement of weight and length.

2.2 Ethical statement

This research was performed in accordance with the Norwegian Animal Protection Act (implemented EU Directive 2010/63/EU). Approval number FOTS ID 5793 was issued on December 12, 2013 by IACUC of Norwegian School of Veterinary Science (since 2014 Norwegian University of Life Sciences, Faculty of Veterinary Medicine and Biosciences, Oslo, Norway).

2.3 Biometric parameters

Weight and length were measured 1.5 years after exposure, in 22 male and 22 female anesthetized fish from both the control and 8.7 mGy/h groups. In the 53 mGy/h group, weight and length were measured in 10 males and 10 females and in 24 fish of undetermined sex. The condition factor of unexposed and gamma irradiated fish was calculated according to the formula ($K = [\text{mass (g)} \times 100]/[\text{length (cm)}]^3$) (Jones et al., 1999).

2.4 Reproduction assessment

Thirty adult irradiated male and female zebrafish of the AB wild type strain were used in the breeding trials. The mating experiments took place during six consecutive breeding weeks one month after gamma irradiation and during five consecutive breeding weeks one year after irradiation. One breeding trial was performed in each week for all groups simultaneously. For maintenance during the reproduction experiments, males and females from each exposure were divided into two groups, kept in 12 holding tanks of 2L volume, with 12 fish per tank and used intermittently over even and odd numbered breeding weeks. In each breeding trial, six standard (conservative) 1L breeding tanks with a meshed bottom for separation of eggs (Aquatic Habitats, Apopka, FL, USA) were used with one breeding pair per tank. The setup and male/female separation took place in the late afternoon and breeding pairs were formed using one male and female from the same exposure group. The morning after, barriers were removed and the breeding pairs were allowed to mate for 30 minutes. Egg collection and counting was performed immediately after breeding, followed by the separation of sexes and transfer of fish to holding tanks.

2.5 Fish anesthesia and euthanasia

For anesthesia of the fish, 0.2% Tricaine Methanesulfonate (MS-222) (Sigma-Aldrich, Oslo, Norway) in dH_2O adjusted to pH 7.0 with 1.0M Tris (pH 9.5) combined with iced system water was used. Briefly, fish remained in this solution until no visible movement was observed. For euthanasia, an overdose of tricaine was used in iced system water, and the fish were observed until failing to react to external stimuli and/or following cessation of opercular (gill) movement.

2.6 Histopathological analysis

Whole fish were fixed individually in 4% paraformaldehyde for a minimum of 4 days and then processed according to standard histological procedures using Hematoxylin and Eosin (H&E) stain. Histopathological examination was performed blindly using a Zeiss Axioskop

159 microscope equipped with a digital camera (Leica SFC 420). Eight males and eight females
160 from the two exposed groups and controls were processed, examined and analyzed 1.5 years
161 after gamma exposure.

162 **2.7 Genotoxicity analyses**

163 ***2.7.1 Comet assay***

164 For blood extraction, eight male and eight female fish were used from the two exposed groups
165 and controls. The fish were euthanized 1.5 years after exposure, and a modified protocol similar
166 to previous studies (Kovács et al., 2015) was used for blood collection for the Comet assay.
167 Briefly, a 200 µl pipette was coated with 10 µl Heparin (5000 IE/ml, Leo®, Norway). After the
168 tail was cut off, 5 µl of blood was collected with the coated pipette and transferred to a
169 microtube containing 100 µl PBS without Ca^{2+}/Mg^{2+} (pH 7.4). Samples were diluted 1:20 with
170 PBS in order to obtain a cell concentration of $1x10^6$ cells/mL. Cell viability was checked by
171 trypan blue exclusion assay. Cells were resuspended 1:10 in 0.75 % low melting point agarose
172 at 37 ºC, and triplicates (3 × 4 µL) from each biological replicate were immediately applied on
173 a cold GelBond®film (as described in Gutzkow et al., 2013). Lysis was performed overnight
174 in lysis buffer at 4 ºC (2.5 M NaCl, 0.1 M Na2EDTA, 0.01 M Tris, 0.2 M NaOH, 0.034 M N-
175 laurylsarcosine, 10 % DMSO, 1 % Triton X-100, pH 10). For unwinding, films were immersed
176 in cold electrophoresis solution (0.3 M NaOH, 0.001 M Na2EDTA, pH > 13) for 40 min.
177 Electrophoresis was carried out in cold, fresh electrophoresis solution at 25 V (0.8 V/cm across
178 the platform) for 20 min at 8 ºC, with circulation of the electrophoresis solution. After
179 electrophoresis, films were neutralized with a neutralization buffer (0.4 M Tris–HCl, pH 7.5)
180 for 2×5 min, fixed in ethanol (> 90 min in 96 % ethanol) and dried overnight. Films were stained
181 with SYBR®Gold Nucleic Acid Gel Stain (Life Technologies, Paisley, UK) diluted 1:10 000
182 in TE-buffer (1 mM Na2EDTA, 10 mM Tris–HCl, pH 8) before examination at a 20 ×
183 magnification under an Olympus BX51microscope (light source: Olympus BH2-RFL-T3,

Olympus Optical Co., Ltd.; camera: A312f-VIS, BASLER, Ahrensburg, Germany). Fifty randomly chosen cells per replicate (150 cells per biological replicate, total 1200 cells per dose rate) were scored using the Comet IV analysis software (Perceptive Instruments Ltd., Bury St. Edmunds, UK). Tail intensity (% Tail DNA), defined as the percentage of DNA migrated from the head of the comet into the tail, was used as a measure of DNA damage to assess genotoxicity (Kumaravel and Jha, 2006). Blood cells were also categorized according to the grade of damage using the % of Tail DNA based on the previously mentioned criteria (Gomes et al., 2013): minimal 10% tail, low damage 10-25%, mid-damage 25-50%, high damage 50-75% and extreme damage >75%.

2.7.2 Blood slide examination

Peripheral blood was obtained from 8-11 males and females from the two exposed and control groups 1.5 years after irradiation. The tail of the euthanized fish was removed and approximately 5 µl of blood was collected by pipette from the severed tail of each euthanized fish, transferred to the frosted end of a glass slide, spread in a thin film and air-dried. After fixation in ethanol for 15 min, slides were left to air dry. The staining was performed using the Quick dip protocol (H&E). The stained slides were viewed under a Zeiss Axioskop microscope equipped with a digital camera (Leica SFC 420) and magnification 1000x, and between 1000-2000 erythrocytes scored per slide. The erythrocytes were also examined for the occurrence of two nuclei (binuclear cells) and for irregular shape (e.g. tear or sickle shaped erythrocytes). The cells with one, two or three micronuclei (MN) were noted separately. Criteria for the identification of fish micronuclei were previously described (Oliveira et al., 2009; Song et al., 2012): (a) MN should be a size smaller (1/10 to 1/30) than the main nucleus (b) MN should be a circular or ovoid chromatin body with the same staining characteristics as the nucleus; (c) MN must not touch the main nucleus.

208 **2.8 Statistical analysis**

209 Statistical analyses were performed using GraphPad Prism 7.02 (GraphPad Software Inc., La
210 Jolla, CA, USA) and XLStat2017® (Addinsoft, Paris, France). Data was tested for normality
211 and homogeneity of variances using Shapiro-Wilk and Levene´s tests, respectively, to check if
212 they satisfy the assumptions associated with parametric tests. Biometric and reproduction
213 parameters, as well as genotoxicity endpoints, did not meet the assumptions of parametric tests,
214 so the non-parametric test of Kruskal–Wallis One Way Analysis of Variance on Ranks was
215 applied to all data. If significant, pairwise comparisons were performed using the Dunn's test
216 to discriminate differences between groups. Results are presented as median (interquartile
217 range). Statistical significance was set at $p < 0.05$.

3 Results

3.1 Biometric parameters in adult zebrafish

The weight and total length were measured in all fish 1.5 years after exposure in order to determine possible differences in size and condition factor (K) between exposed and control fish. Significant reduction of mean length and weight was observed in females of the 8.7 mGy/h exposure group, although there was no difference in condition factor (Table 1). In contrast, the length and weight of males in the 8.7 mGy/h were not significantly different compared to controls, however, the significant difference was found in the condition factor of these males compared to controls (Table 1). No significant differences were however found in fish in the 53 mGy/h group compared to controls (Table 1). For the 53 mGy/h exposure group, external sexual characteristics were non-distinguishable in 40 % of the fish 1.5 years after the exposure, hence this group was excluded from statistical analyses.

Table 1. Biometric parameters in male and female zebrafish measured 1.5 years after exposure to gamma radiation used for the reproduction, histopathology and MN assay. Data are presented as median (interquartile range). Significance compared to corresponding controls denoted with (*) and significance compared to the other exposed group denoted with (**), (Kruskal–Wallis test, $p < 0.001$; Dunn's method, $p < 0.05$).

Dose rate (mGy/h)	Sex	Length (cm)	Weight (g)	Condition factor (K)
Control [a]	male	3.4 (3.3; 3.5)	0.29 (0.26; 0.34)	0.75 (0.67; 0.82)
	female	3.7 (3.47; 3.9)	0.42 (0.36; 0.49)	0.82 (0.78; 0.9)
8.7 [b]	male	3.4 (3.27; 3.5)	0.25 (0.23; 0.29)	0.63 (0.59; 0.69)*
	female	3.5 (3.3, 3.5)*	0.33 (0.28; 0.36)*	0.78 (0.71; 0.83)

	male	3.4 (3.37; 3.5)	0.26 (0.23; 0.32)	0.67 (0.6; 0.79)
53 [c]	female	3.75 (3.6; 3.9)**	0.43 (0.36; 0.46)**	0.8 (0.74; 0.83)
	n.d	3.7 (3.62; 3.8)	0.33 (0.29; 0.37)	0.65 (0.56; 0.69)

K – ([mass (g)*100] /[length (cm)]^3)

[a] N = 22 males, 22 females

[b] N = 22 males, 22 females

[c] N = 10 males, 10 females and 24 fish of no determined (n.d) sex

3.2 Gamma radiation causes reproduction impairment and damage in gonads

The results of the breeding studies indicated a significant reduction in the reproductive capacity of fish exposed to gamma radiation, both at one month and one year after the exposure. The cumulative embryo production per week in the 53 mGy/h group was significantly reduced one month after irradiation, both compared to controls (p = 0.001) and to the 8.7 mGy/h group (p = 0.01) (Fig. 2). One year after exposure, the reduction in embryo production was found to persist in the 53 mGy/h group compared to controls (p = 0.006), as only one breeding pair produced embryos (Fig 2). On the other hand, the cumulative embryo production per week in the 8.7 mGy/h group one month and one year after irradiation did not significantly differ from the control, despite being reduced (~33%) (Fig 2).

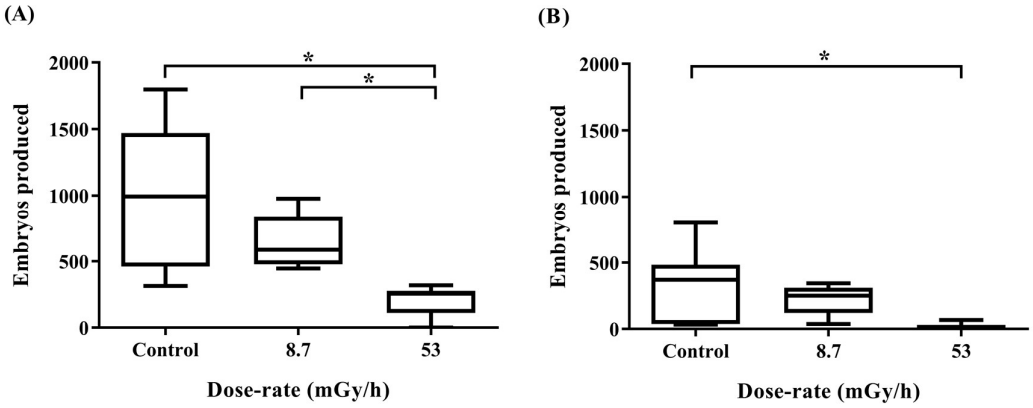


Fig 2. Cumulative embryo production in zebrafish per week one month and one year after exposure to gamma radiation during gametogenesis to either 8.7 or 53 mGy/h compared to controls. The box plots middle line represents the median, the edges delimit the 25th and the

75th percentile, while whiskers indicate the 10th and 90th percentile (Kruskal-Wallis test, $p < 0.002$, Dunn's method, $p < 0.05$). The asterisks indicate significant differences between designated groups (n = 6 breeding pairs per breeding week).

Similarly, embryo production per breeding pair in the 53 mGy/h group differed significantly from the controls and in one trial from the 8.7 mGy/h group one month after the exposure (Table A1). One year after the exposure, the 53 mGy/h significantly differed from the control in two trials (Table A1). In contrast, the embryo production per breeding pair in the 8.7 mGy/h group was not significantly different from the controls (Table A1).

The histopathological examinations revealed significant effects in the gonads of the adult fish (2 years of age). Differences were found between controls and the 8.7 mGy/h females where 62.5 % of females (n = 8) of the latter group had ovaries containing predominantly previtellogenic oocytes (Fig 3B), whereas in the controls the ovaries had oocytes at all developmental stages (Fig 3A). In the 53 mGy/h group, the reproductive organs were massively regressed, which is consistent with the observed failed spawning and lack of embryo production (Fig. 3C).

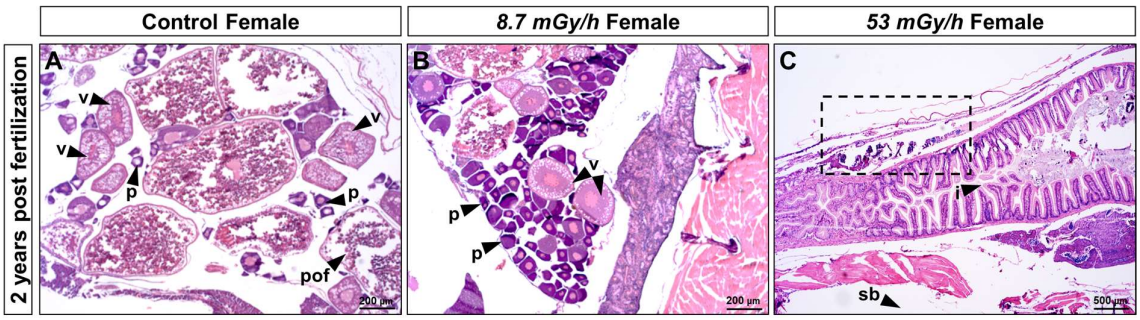


Fig 3. Histological sections of ovaries from (**A**) Control zebrafish with vitellogenic follicles (*v*), previtellogenic follicles (*p*) and postovulatory follicles (*pof*). (**B**) Female zebrafish exposed to 8.7 mGy/h during gametogenesis. Ovaries with a high number of previtellogenic follicules (p); (**C**) Female zebrafish exposed to 53 mGy/h during gametogenesis, showing no visible reproductive organs (dashed rectangle), i – intestine, sb – swimming bladder.

3.3 Persistent genotoxicity

3.3.1 Gamma radiation causes increased DNA damage

DNA damage assessed one year after gamma radiation exposure in whole blood of adult fish using the alkaline single-cell gel electrophoresis (SCGE) assay was significantly higher in exposed groups compared to controls. Males in the 8.7 mGy/h and 53 mGy/h groups showed a 3-fold and 2-fold increase in DNA damage respectively, compared to controls (Fig 4A). Similarly, in females, a 1.2-fold and 3-fold increase in DNA damage was found in 8.7 and 53 mGy/h groups respectively (n = 8 female and 8 male fish), compared to controls (Fig 4B). The DNA damage was also significantly different between the 8.7 and the 53 mGy/h group in both males and females.

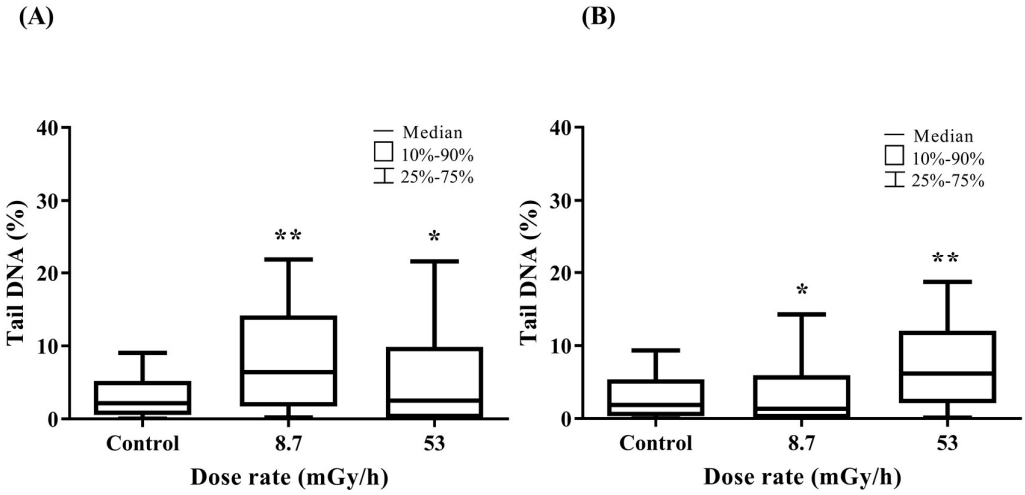
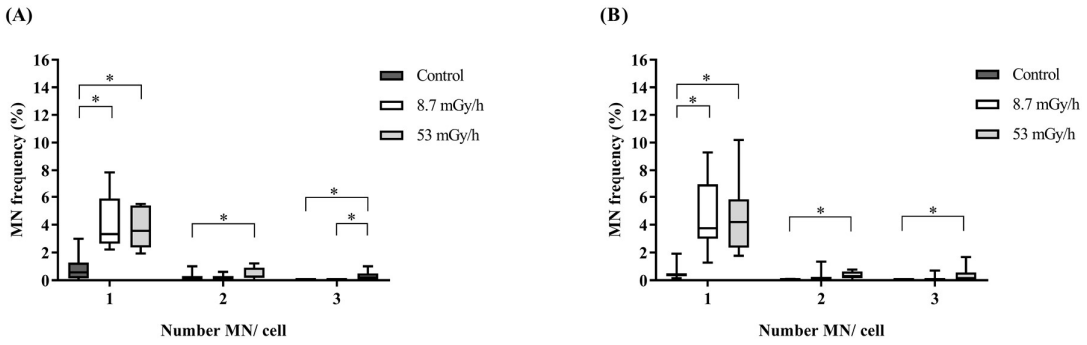


Fig. 4. DNA damage in adult zebrafish measured by the alkaline SCGE after exposure to gamma radiation. Statistical significance between groups denoted with asterisks (Kruskal-Wallis test, $p < 0.001$, Dunn's method, $p < 0.05$; n=1200). (**A**) Male zebrafish (n=8). (**B**) Female zebrafish (n=8).

The percentage of DNA in the tail was used to categorize the grade of damage in unexposed and gamma irradiated zebrafish (Table A2). The majority of cells from both males and females from the control group showed minimal to low grade of damage (> 99% of the cells), characterized by zero or minimal DNA 'Comet-tail'. On the other hand, irradiated zebrafish presented a higher number of cells with low and mid damage compared with the control, reflecting an increase of DNA damage resulting from exposure to gamma radiation.

3.3.2 Gamma radiation causes persistent increase in mitotic malfunctions

Whole zebrafish blood slides were examined in order to determine possible abnormalities related to blood cell formation or renewal. Consequently, micronuclei (MN) were found in erythrocytes, and counts revealed a statistically significant increase in the frequency of one MN per cell in both males and females from the 8.7 and 53 mGy/h exposures, compared to controls ($p \leq 0.0005$) (Fig 5). Two and three MN per cell were found to be significantly more frequent in the 53 mGy/h males and females than in the controls ($p < 0.05$). No significant differences were found in the increase of either micronuclei frequency or the number of MN per cell between the sexes ($p > 0.5$). Furthermore, the occurrence of irregular erythrocyte shape and binucleated cells in the exposed fish compared to controls was examined, without demonstrating any significant difference between the controls and the exposed zebrafish ($p > 0.9$).



315 **Fig 5.** Frequency of micronucleated erythrocytes in zebrafish exposed to 8.7 and 53 mGy/h
316 dose rates (total 5.2 and 31 Gy) of gamma radiation and controls; X-axis shows the number of
317 micronuclei found per erythrocyte. In the box plots, the middle line represents the median, the
318 edges delimit the 25th and the 75th percentile, while whiskers indicate the min and max values
319 (Kruskal-Wallis test, $p < 0.001$, Dunn's method, $p < 0.05$). The asterisks indicate significant
320 differences between different doses in the designated groups of MN frequencies. (n =10.000
321 cells from 8-11 individuals). **(A)** Male zebrafish. **(B)** Female zebrafish.

4 Discussion

4.1 Fish condition and reproduction

This study has shown that exposure to gamma radiation (subchronic, 53 and 8.7 mGy/h, total 31 and 5.2 Gy) during the period of gametogenesis can severely affect the reproduction in fish. The dose rates and doses used in this study are similar to the doses accumulated in the Chernobyl cooling pond reactor, which were up to 10 Gy during the first 60 days of the accident (Hinton et al., 2007) and dose rates to aquatic biota of 12.5 - 33 Gy/h observed in 1957 in Ural lakes near Mayak PA, which resulted in death of the lake ecosystem (Kryshev and Sazykina, 1998). However, the dose rates used in this study are almost two orders of magnitude above the maximum dose rates (130-140 μGy/h) found in the aquatic environment following the Fukushima Daiichi accident (Johansen et al., 2015; Strand et al., 2014). Although the fish survived the exposure, massive pathological changes in the gonads and reproductive failure were found, especially at the higher dose (31 Gy). Gametogenesis is the process in which cells undergo cell division and differentiation in order to form the mature male or female germ cells, which in zebrafish lasts for approximately four weeks between 3- 5 months of age (Koç et al., 2008; Laan et al., 2002). In fish, successful reproduction is dependent upon a good body condition and sufficient energy reserves. As such, condition factor (K) (Jakob et al., 1996; Stevenson and Woods, 2006) was used as an indicator of overall health of fish populations, with heavier individuals of a certain length regarded as being in better breeding condition (Fulton, 1904; Bolger and Connolly, 1989). We found a slight, but significant difference in the condition factor in males exposed to 8.7 mGy/h gamma radiation compared to controls at 1.5 years after gamma irradiation. We also found that the females of the 8.7 mGy/h group were of smaller size, while the condition factor was not significantly different from the other groups. For using the described dose rates and the required number of biological replicates, the fish

were randomly selected for each exposure tank, indicating that individual differences could have been present between fish in different exposures. Since the husbandry of the fish and water parameters did not differ significantly between exposure tanks (Hurem et al., 2017b), the reason behind these differences is unclear, but could reflect the balance between energy budget allocations between growth, repair of DNA damage and spermatogenesis. It is also worth noting that the number of fish in the 53 mGy/h exposure was reduced due to not finding reproductive organs in more than half of the fish (24 fish of undetermined sex). Therefore, it is possible that this confounds the biometric parameter analysis in this group.

A significant reduction in reproductive capacity, in terms of embryo production, was found in the 53 mGy/h group compared to the controls one month after irradiation (this reduction being significantly greater in the 53 compared to the 8.7 mGy/h group) and one year after irradiation. On the other hand, the difference between 8.7 mGy/h group and controls was not significant one month and one year after gamma irradiation. However, oocyte maturation at 1.5 years after gamma irradiation was found to be severely disrupted with only non-mature previtellogenic oocytes predominating in the ovaries in more than half of the 8.7 mGy/h females. Similarly, reduced fecundity and fertility in fish were reported after gamma irradiation of medaka (*Oryzias latipes*) eggs with a dose of 5 Gy (362.5 mGy/h) (Hyodo-Taguchi and Etoh, 1983), while only temporary sterility was induced in medaka after 5 and 10 Gy gamma irradiation (Michibata et al, 1976). Effects on the maturation of oocytes has previously been reported after a whole body exposure of adult loach, *Misgurnus anguillicaudatus* (10 Gy, x-rays), which is approximately two times higher the dose used in our study (Egami and Aoki, 1966). In addition, decreased vitellogenin concentration was found in zebrafish ovaries after exposure to alpha emitters (250 µg/L depleted U for 20 days) (Bourachot et al., 2014). It was earlier established that acute radiation at a dose of 2.5 Gy (X-rays) can impair the gametogenesis in fish, with a 50 % reduction in spermatogonia (Hyodo-Taguchi and Egami, 1976). This study, however, revealed

371 no visible differences in the testis of the 8.7 mGy/h (total 5.2 Gy) exposure group compared to
372 control. Considering the differences observed in ovaries in the 8.7 mGy/h group, the results
373 may indicate that female gonads are more susceptible to gamma radiation than male, as
374 previously suggested by Hyodo-Taguchi and Etoh, (1983). Interestingly, a dose of 4.7 Gy
375 gamma radiation, which is relatively close to the total dose used here, caused accelerated
376 spermatogenesis in medaka according to Kuwahara and co-workers (Kuwahara et al., 2003). In
377 the present study, however, reproduction was severely impaired in fish in the 53 mGy/h
378 exposure group as they produced no embryos one year after the irradiation event, and showed
379 complete regression in ovary and testis development. Additionally, in offspring of the 53 mGy/h
380 exposed fish, modulation of gene pathways related to the endocrine regulation of reproduction
381 was found. These pathways include estrogen receptor 1 (ESR1), follicle stimulating hormone
382 (FSH) signalling, insulin growth factor 2 (IGF2) and gonadotropin releasing hormone (GnRH)
383 signalling (Hurem et al., 2017c). Offspring of these fish (53 mGy/h) also showed 100 %
384 mortality occurring at 8 hours post fertilization (hpf), corresponding to the gastrulation stage
385 (Hurem et al., 2017b). This finding indicates that damaging signals that could lead to a
386 modulation of reproduction hormone pathways, may have been transmitted to the progeny via
387 parental germ cells.

388 **4.2 Genotoxicity**

389 Gamma radiation exposure to 8.7 – 53 mGy/h (total doses 5.2 and 31 Gy) caused a small but
390 significant increase in DNA damage in male zebrafish a considerable time after the irradiation
391 ended (1.5 years), with the most prominent effect occurring in the 8.7 mGy/h exposed males.
392 In females, the DNA damage was significantly increased only in the females exposed to 53
393 mGy/h.

394 It is worth recalling that the numbers of fish in the 53 mGy/h group were reduced due to a high
395 number having undetermined sex. This could confound the results of endpoint analysis in this

group, for example, if the group retaining male traits were in way "more robust" to the radiation challenge. However, with this caveat noted, we feel it is acceptable to include results from this group. The persistence of DNA damage in all the exposure groups may reflect genomic instability, similar to that observed in the progeny of these fish one year after exposure of the parents (Hurem et al., 2017b). However, only a few studies have to date discussed sex-specific differences in sensitivity to ionizing radiation. A study in mice reported higher ionizing radiation induced (1 Gy, X-rays) DNA damage increase in males than in females (Koturbash et al., 2008), and attributed the effect to sex hormones and distinct cellular responses to whole body irradiation, considering that sterilization neutralized this difference. Therefore, it is conceivable that differences in endocrine signaling may contribute to higher susceptibility of male fish to DNA damage.

Although we found no studies in literature on the genotoxic effects of chronic gamma irradiation, DNA damage in whole blood of adult zebrafish was found to be significantly increased after an acute exposure to high doses of ionizing radiation (X-rays, 0.1 – 1 Gy), while DNA damage in the offspring was correlated with the DNA damage of the parents (Lemos et al., 2017). The DNA damage response was also examined after chronic exposure to depleted uranium (20 and 250 µg U/L for 20 days), and differences between males and females were observed (Bourrachot et al., 2014). Interestingly, in offspring of both the 8.7 and 53 mGy/h fish, a high expression of ribonucleotide reductase subunit 2 (rrm2) was found (unpublished data). This gene is associated with DNA damage response in mammals and may perhaps have a role in the transmission of DNA damage to the offspring, in addition to non-targeted mechanisms such as inflammatory and bystander effects following radiation exposure (Hurem et al., 2017b).

Micronuclei originate from aberrant mitosis and are formed when intact chromosomes or their fragments are not properly segregated into the daughter cells nuclei after cell division and

instead remain in the cytoplasm (Pernot et al., 2012; Sabharwal et al., 2015). The MN test is frequently used in fish as an indicator of environmental stress and correlates to increased DNA damage and mutation rate (Russo et al., 2003, Pavlica et al., 2011; Song et al., 2012; Luzhna et al., 2013). In the present study, the frequency of one MN per erythrocyte was significantly increased in the 8.7 and 53 mGy/h groups (males and females) compared to controls. The increase in MN demonstrates mitotic failure indicating a persistent genotoxic stress. It is worth noting that in male zebrafish, the frequency of one MN per cell was higher in the 8.7 mGy/h exposure group than in the 53 mGy/h, while in the females this frequency was higher in the 53 mGy/h than in the 8.7 mGy/h group (Fig 5). Although not statistically significant, the sex-difference in sensitivity in MN-formation resembles the difference in DNA damage increase in the different exposure groups for males and females (Fig 4, Table A2). This supports the fact that the micronucleus test in whole blood seems to be a good indicator of increased DNA damage in zebrafish (Luzhna et al., 2013). The differences in effects between the irradiated groups and control group suggest that genotoxic effects of gamma irradiation during the sensitive period of gametogenesis persist for up to one year after irradiation.

5 Conclusion

The present study demonstrated that subchronic gamma radiation (8.7 and 53 mGy/h) during the gametogenesis stage causes adverse reproductive and genotoxic effects such as increased MN formation in erythrocytes and DNA damage in whole blood persisting 1.5 years after gamma irradiation. Reduced embryo production and disrupted ovary development were found at dose rates ≥ 8.7 mGy/h one month and 1.5 years after the exposure, respectively, while sterility was observed in the highest dose rate (53 mGy/h) one year after exposure, including a total regression of the reproductive organs. Overall, while the doses used in this study did not cause increased mortality of irradiated fish, the observed adverse reproductive and genotoxic

effects indicate that gametogenesis is a very sensitive life stage to ionizing radiation exposure and that the difference in effects can be sex-dependent and transmissible to offspring.

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